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The role of the IFT machinery in the construction of the eukaryotic flagellum/cilium

Role IFT v procesech stavby eukaryotního bičíku/řasinky

Bachelor´s thesis

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Prohlášení:

Prohlašuji, že jsem tuto bakalářskou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literatuře. Tato práce ani její podstatná část nebyla předložena k získání stejného nebo jiného akademického titulu.

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Content

Abstract

Intraflagelar transport (IFT) is a bidirectional continuous process providing growth, maintenance and remodeling of eukaryotic cilia and flagella. The cilia and flagella are microtubular-based organelles with several functions such as signalling and motility. Building blocks of the ciliary cytoskeleton are produced in the cell body and needs to be transported to the distal end, which is the sole place of their assembly. This transport is facilitated by the IFT complexes, which are carried from the cell body along the microtubules towards the distal end by kinesin motor protein. Subsequent recycling of the IFT units as well as turnover of ciliary building blocks is facilitated by dynein powered movement towards the cell body. The regulation of this process is still unknown. While composition of the IFT machinery has been characterized, the processes related to IFT switch from distal-end directed to the proximal-end directed, which happens at the ciliary tip, are largely unknown. Another outstanding question concerns how is the IFT regulated in order to achieve a defined length of the cilium. This thesis briefly examines the structure of cilium, composition of the IFT machinery and the processes occurring during the transport and discuss several possible models of IFT regulation.

Keywords

axoneme, cilium, dynein, flagellum, kinesin, microtubules

Abstrakt

Intraflagelární transport (IFT) je obousměrný kontinuální proces, který zajišťuje růst, údržbu a remodelaci řasinek a bičíků. Řasinky a bičíky jsou organely, které jsou založeny na mikrotubulární struktuře. Mezi jejich hlavní funkce patří signalizace a generování pohybu. Proteiny určené ke stavbě řasinkového cytoskeletu jsou produkovány v těle buňky a musí být transportovány na distální konec řasinky, což je jediné místo, kde jsou do cytoskeletu zabudovány. Tento transport je zprostředkován pomocí IFT komplexů, které jsou přenášeny z těla buňky podél mikrotubulů směrem k distálnímu konci pomocí molekulárního motoru kinezinu. Následná recyklace IFT jednotek a nahrazování proteinů určených k dostavbě řasinkového cytoskeletu jsou umožněny dyneinem poháněným pohybem směrem k tělu buňky. Regulace tohoto procesu je stále neznámá. Zatímco kompozice IFT aparátu již byla charakterizována, procesy související s přepínáním IFT z pohybu směrem k distálnímu konci na pohyb k tělu buňky zůstávají neznámy. Další významnou otázkou je regulace IFT ve vztahu k dosažení správné délky řasinek. Tato práce krátce popisuje strukturu řasinky, kompozici IFT aparátu a procesy, probíhající v rámci transportu a hovoří o několika možných modelech regulace IFT.

Klíčová slova

axonema, bičík, dynein, kinezin, mikrotubuly, řasinka

List of abbreviations

ATP	adenosine triphosphate
IFT	intraflagellar transport
GTP	guanosine triphosphate
MAPs	microtubule-associated proteins
ODA	outer dynein arms
IDA	inner dynein arms
DRC	dynein regulatory complex
HC	heavy chain
LIC	light intermediated chain
KHC	kinesin heavy chain
KLC	kinesin light chain
TFs	transition fibres
FTC	flagellar tip complex
Cryo-ET	cryo-electron tomography
EM	electron microscopy

1. Introduction

The cilium, also known as the flagellum (the terms are used interchangeably) is an evolutionary conserved microtubule-based organelle. Two basic types of cilia are known – primary cilia and motile cilia. Both possess the sensory function, providing the information about changes in the extracellular environment for control of homeostasis and tissue development. Moreover, the motile cilia facilitate motility. They either power movement of individual cells, such as sperms or transport of fluid over the epithelia, for instance, in the respiratory system or cerebrospinal fluid spaces (reviewed by: Pedersen et al., 2012).

The cilia in general possess nine microtubular doublets, arranged in a circle – the axoneme. This structure acts as a scaffold for additional components of the cilium. The primary non-motile cilia, such as in kidney tubule cells, fibroblast or neurons (D'Angelo & Franco, 2009) typically have this 9+0 arrangement. The motile cilia possess in addition a pair of microtubules in the centre. This arrangement is known as "9+2" cilia (Satir & Christensen, 2006). However, a number of exceptions to this pattern are known across species. For instance, the notochordal plate cilia in rabbit embryos have "9+4" axonemal arrangement (Feistel & Blum, 2006).

Force generating and doublet movement coordinating structures are present in exact positions on the microtubules. The beating movement is provided by sliding of doublets on one side while doublets on the other are relaxed (Figure 2A). This is enabled by several structures, which enable communication of opposing doublets via the central pair (Lindemann & Lesich, 2010). A large number of ciliary proteins are necessarily for a fully functional motility. Mutations in genes coding for ciliary components lead to a wide range of genetic diseases, generally called ciliopathy (reviewed by: Pedersen et al., 2012). Ciliopathies manifest in large variety of pathological phenotypes affecting different organs and organ systems (Braun & Hildebrandt, 2017). Because in the cilium ribosomes are absent (Lechtreck, 2015), all ciliary material must be transported from the cell body. As the axonemal assembly occurs strictly at its distal end. (Marshall & Rosenbaum, 2001) the ciliary building blocks need to be transported from the cell body to the distal end of the cilium. This transport is facilitated by so-called intraflagellar transport IFT) (Marshall & Rosenbaum, 2001).

The IFT is required for the growth of the cilium and after the cilium reaches its mature length, IFT is also required for its maintenance and remodelling (Marshall & Rosenbaum, 2001). TIFT is continuous, bidirectional transport (Figure 1), powered by molecular motors, kinesin-2 and cytoplasmatic dynein. Kinesin-2 a microtubular based plus end-directed motor, moves in the anterograde direction from the cell body towards the distal end along the B-tubule of outer doublet

(Kozminski et al., 1995). Dynein moves in the retrograde direction from the distal end towards the cell body along the A-tubule of outer doublet (Pazour et al., 1998). IFT motors carry the IFT complexes, which are effectively connected by an adaptor complex. Together, they are attached with a cargo, which is released upon arrival of IFT complexes at the respective location (Wren et al., 2013).

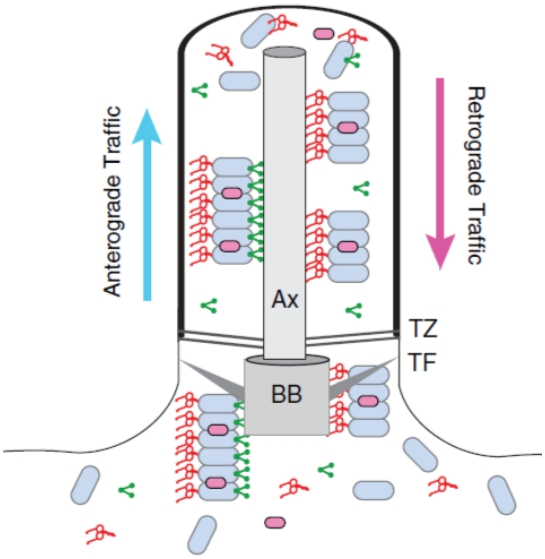

Figure 1: Schematic presentation of IFT
The axoneme (*Ax*); protruding forms the basal body (*BB*); transition zone (*TZ*); transition fibres (*TFs*); IFT kinesins (*green particles*); IFT dyneins (*red particles*); IFT subcomplexes (*blue particles*); BBS proteins (*pink particles*), which are suggested to play key role in the connection between IFT train subunits (Williams et al., 2014). IFT kinesins carry cargo and IFT dyneins to the axonemal tip, where the kinesins are turned off. Here, the material together with molecular motors disassociate from the axoneme, IFT particles reassemble and then dyneins carry cargo retrogradely. Kinesins stays dissociated and return by the diffusion to the cell body. (adapted from Lechtreck et al., 2017)

2. Composition of Cilia

The cilium itself consists of two major cytoskeletal structures – the basal body, which is surrounded by the cytoplasm and the axoneme. The principal components of both structures are microtubules. A large number of specific proteins associate with microtubules of each structure, which is essential for the function of the organelle (Avidor-Reiss et al., 2017).

2.1. Microtubules

Microtubules are cytoskeletal filaments present in all eukaryotes. They are composed of two proteins, α-tubulin and β-tubulin, which are well conserved across eukaryotes. α-tubulin and β-tubulin form heterodimers, that polymerize in a head to tail fashion to constitute a microtubule protofilament. These interact laterally to give rise to a closed tube, the microtubule (reviewed by: Lansbergen & Akhmanova, 2006). Tubulin subunits exchange only at microtubular ends, the fast-growing end (plus end) and slowly-growing end (minus end) (reviewed by: Lansbergen & Akhmanova, 2006). Tubulin polymerization depends on the presence of GTP. (Janke & Bulinski, 2011).

For the purposes of the ciliary function, tubulin is post-translationally modified. Most of the modifications are targeted to the C-terminus of the tubulin subunits. This modifications generally produce the alternation of binding properties, which can lead to affection of microtubule-associated proteins (MAPs), movement of IFT complexes and stability of the axoneme. (Howes et al., 2013). Tubulin can be modified with numerous types of posttranslational modifications, occurring at the C-terminus, such as tyrosination, detyrosination, polyglycylation, polyglutamylation and palmitoylation (Janke & Bulinski, 2011). An additional modification, tubulin acetylation, is not C-terminus specific. In some protists, which possess only a single set of genes for α- and β-tubulin the posttranslational modification are the only mechanism for generating of microtubular diversity (reviewed by: Westermann & Weber, 2003).

2.2. Basic Structures of Cilium

The key role in the process of building the axoneme plays a barrel-like structure known as the basal body., It is formed by modification of centrioles. Around the S phase of the cell cycle, the centrioles take part in formation of the mitotic spindle. Each centriole is duplicated after mitosis (Nigg & Stearns, 2011). The old, mother centriole and the new, daughter centriole together create the centrosome. (Azimzadeh & Bornens, 2007). These two centrioles vary in age and maturity, and consequently have different functions. Only the older, mother centriole, is able to induce the formation of an axoneme and thus becomes a basal body (Nigg & Stearns, 2011). To the mother centriole two classes of appendages are attached - subdistal appendages for the purpose of anchoring microtubules and distal appendages providing the docking of the basal body to the plasma membrane (Gupta et al., 2015). Multiciliated cells with more (usually hundreds of) cilia need to employ two parallel pathways for faster

basal body generation. First of them is a centriole-dependent pathway, which needs pre-existing centrioles as a template (the same process as in cells with a single cilium). The second pathway is called deuterosome-dependent pathway; the basal bodies are created *de novo* without the template. In this case new centrioles – deuterosomes are prearranged around non-microtubular structure (reviewed by: Shahid & Singh, 2018). The major role of the basal body is nucleation of microtubules enabling the construction of the axoneme (Fisch & Dupuis-Williams, 2011). From the basal body 9 microtubular triplets, each composed of microtubule A, B and C elongates. While the A-tubule is complete, the B-tubule (which is accessory to the A-tubule) and the C-tubule are incomplete (Figure 2). In the proximal part of the flagellum, so-called transition zone, C-tubule terminates, leaving A- and B-tubules to constitute the skeleton of the axoneme (reviewed by: Ishikawa, 2017). In motile cilia immediately above the transition zone two singlet microtubules of the central pair initiate. (reviewed by: Fisch & Dupuis-Williams, 2011).

The transition zone, which is selectively permeable, separates the cilium from the cytoplasm and the cytoplasm membrane. The transition zone enables entering proteins designated for the cilium and prevents the cytoplasmatic proteins from entering into the cilium. (Avidor-Reiss et al., 2017), which facilitates composition of the organelle to be different to the rest of the cell.

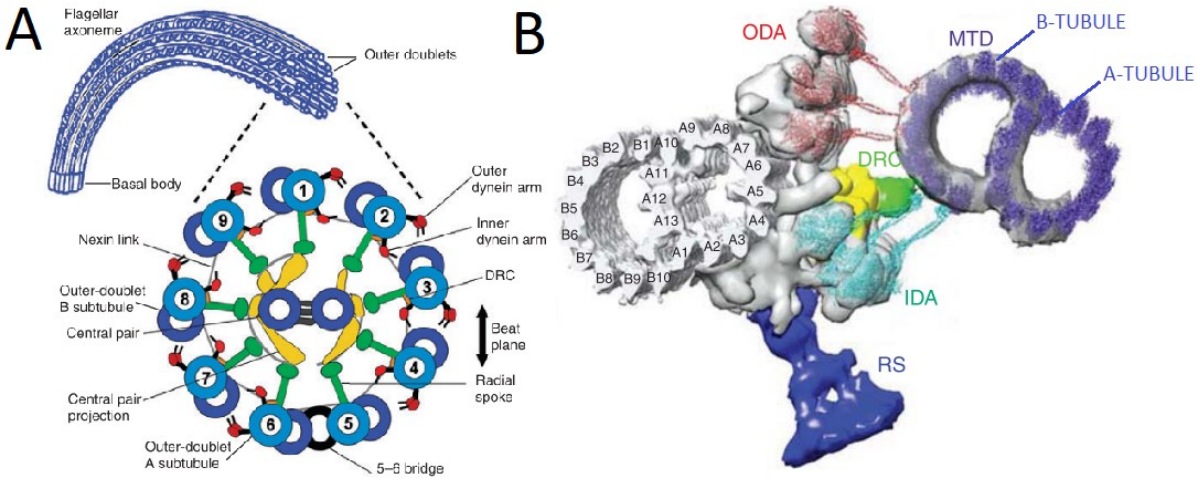

Figure 2: Schematic diagram of the axoneme with major structures shown
(*A*) Scheme of axoneme of a motile cilium in cross section. All components together cooperate in process of axoneme bending. (adapted from Lindemann & Lesich, 2010). (*B*) Atomic model from cryo-ET and single/particle analysis of single outer doublet with associated structures and their binding to microtubules of neighbouring doublets consisting from numbered protofilaments. Radial spokes (*RS*); inner dynein arms (*IDA*); outer dynein arms (*ODA*); dynein regulatory complex (*DRC*); microtubular doublets (*MTD*) with complete A-tubule and incomplete B-tubule. (adapted from Ishikawa, 2017)

Ciliogenesis requires also the transition fibres (TFs), which are localized at the ciliary base in the form of a propeller with nine paddles. The role of TFs mediates the docking of the mother centriole to the membrane and at the same time docking of vesicles containing ciliary cargo (Wei et al., 2015).

2.3. The Axoneme

The crucial part of the cilium is the axoneme. It is a structure made of nine microtubular doublets, which forms the cylinder-like structure. Large number of proteins attached to these doublets are known. Typically, ciliated cell types possess a several micrometres to tens of micrometres long axoneme, which is approximately 300 nm in diameter. Motile cilia in wide range of species have a microtubular pattern “9+2," which refers to nine doublets surrounding the central pair of microtubular singlets as mentioned above. (reviewed by: Ishikawa, 2017).

Every 96 nm the axoneme displays identical repetition of its segment. Adjacent doublets are connected by nexins links, contributing to the cilia motility by connecting the doublets. Motility, caused by bending of the individual doublets is enabled by cooperation amongst nexins, outer dynein arms and inner dynein arms arranged laterally around doublets and connected to the A-tubule. (Figure 2) (Heuser et al., 2009).

Dyneins in general are minus-end-directed molecular motors, enzymes converting chemical energy into mechanical work. There are two groups of dyneins - cytoplasmatic and axonemal. This paragraph deals with the axonemal dyneins; the cytoplasmatic dyneins will be described later. The axonemal dyneins use this energy specifically to power doublet sliding (Wickstead & Gull, 2007). Dyneins are organized into larger complexes, which consist of 1 to 3 dynein heavy chains, each containing the motor domain. Heavy chain is connected to the smaller light, intermediate and light intermediate chains. (Wickstead & Gull, 2007).

Two rows of dynein arms contribute to the axonemal beat generation – outer and inner dyneins, which are both attached to the A-tubule of microtubular doublet and reversibly binds to the B-tubule of a neighbouring doublet (King, 2016). This dyneins are anchored in lines by their N-terminal tails. The adjacent microtubule doublets are shifted with respect to each other, generating the beating motion (reviewed by: Ishikawa, 2012). The importance of inner dynein arms (IDA) and outer dynein arms (ODA) (Figure 2) was demonstrated for example in a *Chlamydomonas reinhardtii*-strains mutated in a respective dynein gene. Mutants show abnormal frequencies of axoneme beating together with significantly decreased velocity of a single beat (Hayashibe et al., 1997). The beating was not

completely stopped, probably due to the presence of many proteins in a dynein complex, which can functionally partly substitute the missing or aberrant protein.

Dynein regulatory complex (DRC) is one of the major regulators of axoneme bending. It is composed of two domains. The basal plate, providing the attachment to the A-tubule and the linker, which spans to B-tubule of a neighbouring microtubular doublet (Heuser et al., 2009). By sensing a position of the adjacent doublet DRC regulates the dynein function and therefore the beating of the cilium (Kozminski et al., 1995).

Radial spokes are composed of approximately 20 polypeptides. These are organized into stalk and head subunits. These subunits are pre-assembled in the cell body and then transported to the site of the axonemal assembly by the IFT (Qin et al., 2004). Radial spoke is completed, when attached to the axoneme (Qin et al., 2004). When dyneins on one side are attached and exert force, the ones on the other side needs to relax. The communication happens via radial spokes and the central pair. This involves phosphorylation but can be replaced by external force. The connection between the central pair and the radial spokes enable the dynein function, providing the axonemal bending. (Warner, 1970). This was also supported by the observation of *Chlamydomonas reinhardtii* mutant in radial spokes proteins exhibiting slow, irregular movement. ODA were able to be activated even without presence of IDA or a central pair. On the other hand, IDA requires presence of central pair for its function. Deletion of central pair in a *Chlamydomonas reinhardtii* leads to significantly slower beating frequencies than wild type. This observation demonstrated, that even when ODA is structurally more similar to IDA, a more important communication happens between IDA and central pair. For the illustration, while wild type beat lasts approximately 0.02 seconds, mutant lacking the central pair displays only one beat per more than one second. On top of, the beating is arrhythmic (Hayashibe et al., 1997).

2.4. Ciliary Tip

The ciliary tip is the region, where the growth of the axoneme exclusively occurs and the turnover takes place. It is also the place where the switch from anterograde to retrograde IFT transport occurs. The pattern of microtubule termination varies greatly between species – A - and B - microtubules and central pair terminates at different position. (Croft et al., 2018). In general, B-tubules in most species end closer to the cell body while A -tubules, together with central pair continue further. The end of A-tubules and the end of the central pair usually contain some kind of capping structures (Croft et al., 2018).

The distal tip of a *Chlamydomonas reinhardtii* flagellum shows a big central pair capping (Figure 3; model 1). Moreover, smaller carrot-like plugs protrude into the A-tubules lumen of outer doublets. The plugs are connected to the ciliary membrane by a distal filament. Presence of the central cap was also demonstrated in *Tetrahymena thermophila*, where the central pair elongated significantly further than the A-tubules (Figure 3; model 2). (Dentler, 1980). In certain organisms such as *Kinetoplastidae*, all microtubules terminate at the very axonemal tip, capped by one big capping structure (Varga et al., 2017) (Figure 3; model 3); The carrot-like plugs are present (Woolley et al., 2006). In mammalian sperm flagellum, individual singlets terminate at different distance from the ciliary tip and are capped with separate structures (Figure 3; model 4). The outer doublets in this case can split and form two complete singlets called "duplex." In the axonemes of the rodents, the duplexes are connected at their tips and thus are indiscernible from the central pair (D. M. Woolley & Nickels, 1985). Mammalian tracheal cilia possess yet different capping structure. A-tubules of the outer doublets and central pair extend and are connected into one capping structure (Dentler & LeCluyse, 1982). The carrot-like plugs are probably anchoring both A-tubules and central pair (Figure 3; model 5). The ciliary crown extends from the ciliary cap trough the cytoplasmatic membrane and forms hair-like protrusions above the membrane, (reviewed by: Croft et al., 2018) which may play role in interactions with mucus. This theory is supported by the presence of ciliary crown – it was found only in duct epithelia (W. L. Dentler & LeCluyse, 1982).

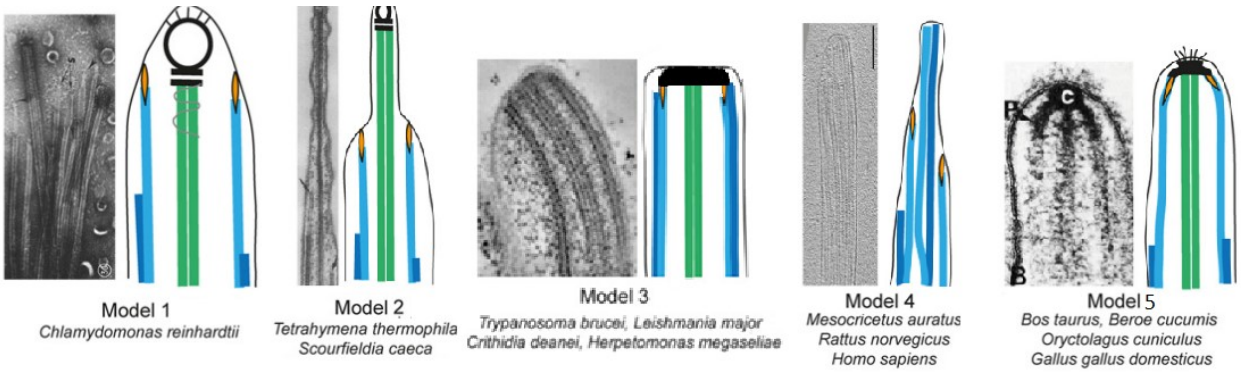

Figure 3: Schematic drawing illustrating six different models of axonemal tips
The highlighted structures are A-tubules (*turquoise*), B-Tubules (*blue*), central pair (*green*), carrot-like plugs (*orange*). Model 3 shows the capping structure, capping together the central pair and both outer doublets (D. Woolley et al., 2006). Model 4 display the mammal sperm axoneme and Model 5 display the mammalian tracheal tissue axoneme (adapted from Croft et al., 2018)

Hence, a common feature of the ciliary tip is presence of microtubule capping structures. Proposed roles of these structures include regulation of microtubule dynamics, IFT turnover, and limiting microtubule sliding, which is essential for the axonemal beat (Lindemann & Lesich, 2010) . Moreover,

there are also roles specific for a particular type of cilia, such as the movement of mucus by respiratory cilia possessing the ciliary crown (Kuhn & Engleman, 1978). This may account for the large variability in morphology of the capping structures. As the protein constituents of the capping structures are largely unknown it is challenging to test their roles experimentally.

3. Ciliary Growth on the Distal End

In *Tetrahymena*, the ciliary growth undergoes two main phases. Early phase, where the fast length extension occurs and the phase, where the length does not change much, yet the tip region development continues. This maturation goes on for several hours after the axoneme reaches the mature length. The capped central pair microtubules reach the mature length prior to the outer microtubular doublets and during the growth push the ciliary membrane. Thus, the capping structures may influence the faster extension of the central pair (Reynolds et al., 2018).

As demonstrated by experiments with *Tetrahymena*, the distal end displays morphological transformation during development. This transformation does not stop when cilium reaches the mature length. The cryo-ET confirmed diverse tip morphologies during the mature state, growth and re-growth of the central pair and the outer doublets (Reynolds et al., 2018).

4. Intraflagellar transport

A fast, bidirectional movement of granule-like particles was observed along the cilium (Figure 4). When originally discovered in 1993, it was apparent, that there are two types of movement – an anterograde (from the cell body to the flagellar tip), which occurred with the speed of approximately 2.0 µm/s, and a retrograde movement from the tip to the cell body, with the speed of approximately 3.5 µm/s (Kozminski et al., 1993). These particles, which are known as IFT trains move between microtubular doublets and ciliary membrane. (Pigino et al., 2009). The IFT train consists of many particles, which themselves consist of biochemically separate complexes – IFT complex A and IFT complex B (Cole et al., 1998). IFT complex A associates with IFT dynein and IFT complex B with kinesin-2. The trains are used for transport of material, which is used for the construction or molecules with signalling roles. (Cole et al., 1998). The cryo-ET images shows that the anterograde trains have a denser structure than the retrograde trains (Jordan et al., 2018). Recently, it was demonstrated that trains moving in opposite directions do not collide because the anterograde IFT trains move along B-tubules

while the retrograde trains along A-microtubules. However, trains heading the same way may interact, whereby a faster train caught up with a slower train and then both of them continued with the same velocity (Stepanek & Pigino, 2016).

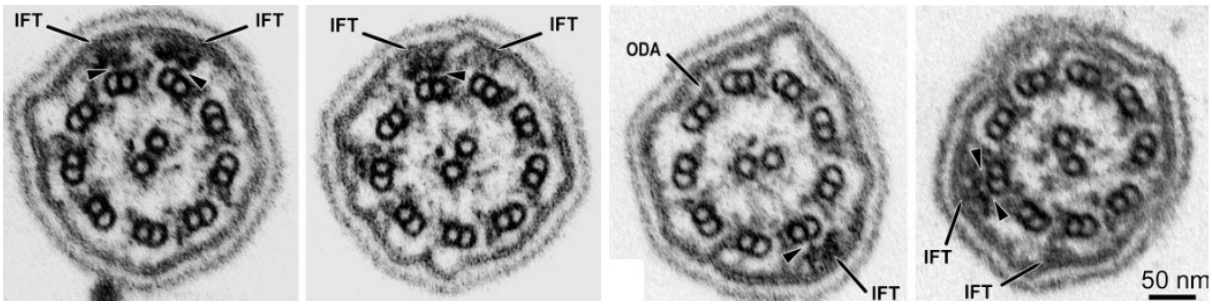

Figure 4: TEM micrographs of flat-embedded flagella from wild type *Chlamydomonas reinhardtii* cells containing IFT trains
Cross-section of the axoneme with visible IFT trains (*IFT*) between flagellar membrane and B-tubules of microtubular doublets close to the outer dynein arms (*ODA*). (adapted from Pigino et al., 2009)

The anterograde trains are composed of active kinesin-2, which carries all other components along the axoneme towards the plus end, IFT complex A, IFT complex B (Pedersen et al., 2006) and many inactive dynein molecular motors (Jordan et al., 2018).

IFT not only play role in cell motility, but also provide transfer into the cell body, which can conclude in cell signalling function, development and controlling of gene expression. The ciliary membrane binds signal receptors and complexes, which are used for transport of signal from the cilium int the cell body. For the This hypothesis is strongly supported by observation of the *Caenorhabditis elegans* mutants, because the direction of their movement is controlled by chemotactic molecules. For instance, the *Caenorhabditis* moves away from the environment with high osmolarity. Mutants in genes for the cilia lead to defects in osmotic avoidance. On top of, The Caenorhabditis also employ the signalling molecule for communication between individual Caenorhabditis, such as mating. Mutants in kinesin-2 motor proteins not initiate the regular signal cascade, which prevents their attraction and thus the mating (Scholey & Anderson, 2006).

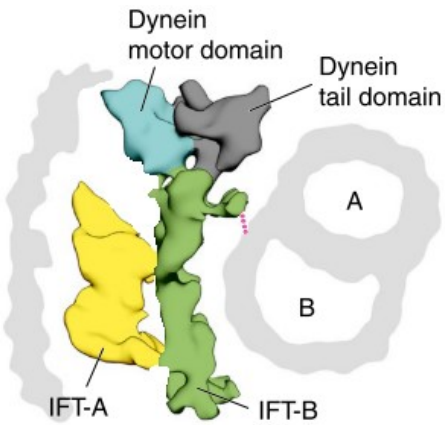

Figure 5: Composition of anterograde IFT train
Representative position of IFT complexes (A and B), dynein domains, microtubular doublets and the membrane (*grey*). Connection between IFT train and B-tubule of microtubular doublet provided by kinesin motor protein is here presented as pink connection (adapted from Jordan et al., 2018)

4.1. Kinesin and the anterograde IFT

The anterograde IFT is delivered by kinesin-2, a microtubular-based motor using energy from ATP hydrolysis. It consists of two motor subunit - kinesin heavy chains, that provides the hydrolysis. Kinesin heavy chains are connected to two kinesin light chains intended for the cargo binding (reviewed by: Scholey, 2013).

In *Chlamydomonas,* when anterograde IFT is inhibited by kinesin inactivation, both anterograde and retrograde IFT transport vanishes. This leads to reduction of the axoneme and no flagella are built (Kozminski et al., 1995). This propose, that the retrograde transport is depended on anterograde trains.

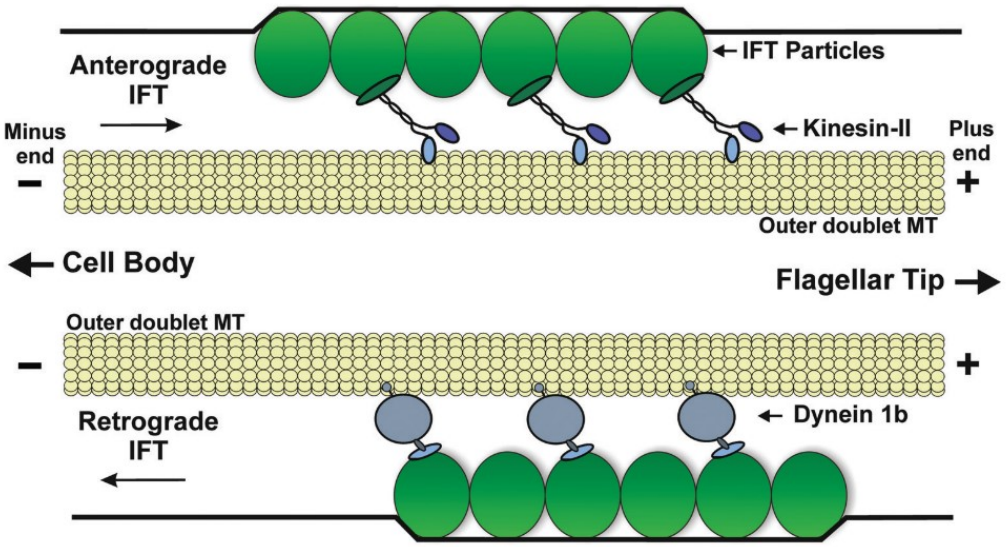

Figure 6: Intraflagellar transport
Anterograde trains are transported towards the plus end of axonemal microtubules by kinesin-2. Retrograde trains are heading towards the minus end, which is facilitated by dynein. The locomotion is continual between the end of the axoneme. The ciliary membrane is elevated when the particles are passing by. (adapted from Cole, 2003)

4.2. Dynein and the retrograde IFT

Cytoplasmatic dyneins are molecular motors, able to walk along the microtubules from their plus end toward their minus end. They also possess the capability to convert ATP into mechanical energy the same way axonemal dynein does, but here it is used for transport of cargo rather than sliding microtubule doublets. Phylogenetically, the IFT dynein motor complex belong to the group of cytoplasmatic dyneins, despite the fact, that they operate within the cilium (with the second group being the axonemal dyneins responsible for the axonemal beat). The dynein motor complex is composed of two heavy chain (HC), each of which possesses a motor domain and several associated chains (Toropova et al., 2017). The microtubule binding occurs at the tip of anti-parallel coiled coils, the stalks. On the opposites side the linker domain is located, which presumably transmits the force between the motor domains and to cargo (reviewed by: Toropova et al., 2017) The light intermediate chain subunits are required for the stabilisation of dynein (Houet al., 2004).

The synthesis of dynein complex is located in the cytosol. The synthetized protein adopts inhibited configuration, linkers are trapped, and stalk crossed. In this state, dynein complexes are transported to the ciliary tip by kinesin-2. Moreover, IFT dynein motors, when transported by anterograde trains,

are located between IFT complex B and the membrane (Figure 5), which also prevents any interaction with the axoneme. When the IFT trains reach the ciliary tip, they must be restructured to form retrograde trains. Dyneins must be repositioned and activated. This hypothesis is supported by cryo-ET observation of separated dynein motor domains at the axonemal tip. The mechanism, how is this IFT remodelling facilitated remains unknown (Jordan et al., 2018). When retrograde transport does not work properly, the anterograde molecular motors are unable to travel back to the cell body and might accumulate in the cilium (Pazour et al., 1998).

For a better insight and understanding of significance of retrograde transport, experiments with LC8 protein will be described. LC8 in a component of dynein light chain (Pazour et al., 1998). Deletion of a *Chlamydomonas reinhardtii* LC8 leads to stopping of the retrograde IFT. Intriguingly, under these conditions the flagellum was not completely abolished; the axoneme was present but shorter and the flagellum was paralyzed (Pazour et al., 1998). Moreover, an abnormal accumulation of particles was observed between microtubular doublets and the flagellar membrane (Kozminski et al., 1993). LC8 mutants occasionally had swellings on the sides or tips of their flagella, which is seemingly caused by accumulation of these particles, which can be explained by inability of retrograde transport. Some particles were connected to the microtubular doublets and some to the flagellar membrane. In addition, in LC8 mutant cells radial spokes and dynein arms were missing and were not observed even in an unassembled state. This may be explained due the fact, that LC8 also a constituent of these structures. (Pazour et al., 1998).

4.3. IFT complexes

IFT complexes facilitate the interaction between bidirectional molecular motors and ciliary cargos. Many of the constituents of these complexes are essential for construction of the cilium; when mutations occur, this has serious consequences for IFT, and leads to severe ciliopathies. Mutation of other auxiliary constituents are not so severe. Both groups are highly evolutionary conserved. (reviewed by: Bhogaraju et al., 2013). IFT constituents are rich in protein-interacting motifs (Cole, 2003).

Depletion of proteins belonging into the IFT complex B causes comparable phenotypes as the depletion of kinesin-2 - decreased or even abolished cilium assembly. The depletion of IFT complex A causes accumulation of IFT B proteins and retrograde cargo at the tip, which is also typical for mutants of retrograde IFT dynein motor proteins. However, in contrast to IFT dynein mutants, IFT A depletion

also causes shortening of the axoneme. It indicates, that one of the functions of IFT A complex might be association of IFT B particles the to the IFT dyneins (Jordan et al., 2018). Otherwise, it could be involved in proper IFT turnover at the ciliary tip. Another possibility suggests the role of the IFT A complex in anterograde transport of the crucial retrograde-associated proteins, which also correlates with the localisation of IFT A complex (Figure 5).

The localisation of IFT B complex implies imply, that its function is to bind together IFT A complex, IFT dynein complex and cargo and serve as the structural core of the anterograde train (Figure 5) (Jordan et al., 2018).

4.4. Axonemal turnover

In the process of ciliary growth, it is necessary to transport thousands of ciliary precursor proteins to the ciliary tip for assembly. The turnover of axonemal material at the axonemal tip occurs and hence old axonemal proteins are transported to the cell body (Qin et al., 2004). This is supported by fact, that even steady-state length of ciliary microtubules is IFT-dependent. Another indication proving the turnover was provided by the experiment, in which two biflagellate *Chlamydomonas reinhardtii* gametic cells fused into a dikaryon form with four flagella. As one of the gametes was expressing HA epitope-tagged tubulin two of four flagella of the quadriflagellate cell initially contained HA tagged tubulin. After some time, the tagged subunits appeared specifically at the tip of the remaining two flagella. This shows, the axoneme remodelling is happening at the axonemal tip, and its subunits can be reused for the same purposes, which may be even assembly of a different axoneme (Marshall & Rosenbaum, 2001).

The journey of the anterograde train starts near the basal body, where the cargo is associated with the kinesin-2 (Figure 7A). The IFT train is then transported along the axoneme towards the plus end of the B-tubule by kinesin-2. The kinesin-2 is attached to inactive IFT dynein (Figure 7B) (Jordan et al., 2018). When the trains reach distal end of the axoneme, they must be remodelled for their competency for retrograde IFT. The observation of IFT trains in *Trypanosoma brucei* shows approximately 1.8 seconds period during which the particles are stationary at the axonemal tip, which is he period needed for particle turnover (Wren et al., 2013). During this period the cargo is likely released from IFT train (Figure 7C) (Wren et al., 2013). Firstly, the IFT complexes A and B are dissociated and IFT dynein is released. After that, IFT dynein is activated. Subsequently, complex A is bound to the active IFT dynein through LIC domain; complex B is then bound to the complex A (Figure 7D). IFT dynein

carry the rest of IFT train retrogradely back towards the basal body and the cell body (Figure 7E), where IFT components and cargo can be recycled (Figure 7F) (Jordan et al., 2018).

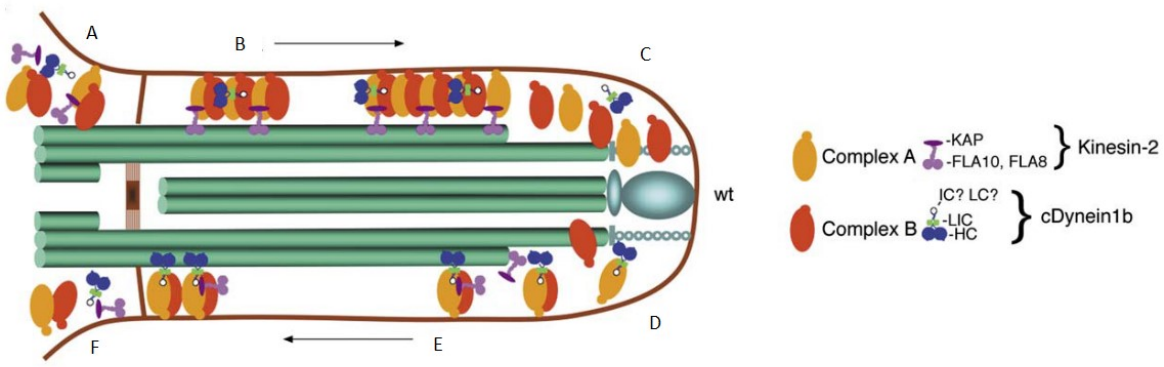

Figure 7: Model for the IFT in *Chlamydomonas*
(*A*) Assembly IFT complexes with motors in the peribasal body region. (B) anterograde transport facilitated by kinesin-2 of IFT complexes A, B and inactive IFT dynein. (*C*) Dissociation of all components of the IFT train. (D) Binding of complex A to the IFT dynein; binding of complexes A and B; binding of kinesin-2 to active IFT dynein.[1] (*E*) Several hypotheses present a model, when return of the kinesin-2 is provided by active IFT dynein, which carries both IFT complexes and kinesins back to the basal body. (*F*) Components of IFT are recycled to the basal body. (adapted from Pedersen et al., 2006)

The fact, that incorporated tubulin does not stay stable in the same place but can be transferred back to the cell and reused raises the question, how exactly is the tubulin subunit removed from the axoneme. There were two possibilities – the subunit removal takes place either at the plus end, same as its incorporation (Figure 8A), or the disassembly occur at the minus end, by the so called treadmilling (Figure 8B). To elucidate this process, biflagellate form of *Chlamydomonas* with half-length flagella was mated with another full-length biflagellate form of *Chlamydomonas*, which was expressing HA epitope-tagged tubulin. After mating, two half-length flagella of quadriflagellate form elongated to full length. Thereafter – if the plus end only model was correct the proportion of tagged and untagged tubulin regions would be unchanged, because only the labelled tubulin at the plus end would be exchanged. The treadmill model on the other hand postulates shortening of the unlabelled tubulin region, as the labelled tubulin would be incorporated at the plus end and the unlabelled tubulin removed from the minus end. In the experiment no shortening of the unlabelled segment was observed, supporting the plus end only model of turnover. (Marshall & Rosenbaum, 2001).

[1] Several authors (reviewed by : Lechtreck et al., 2017) proposed the model of kinesin return by diffusion instead of being transported by dyneins.

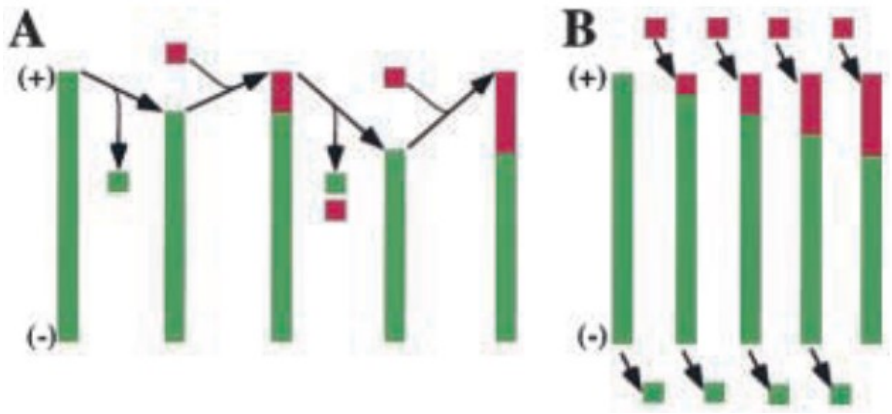

Figure 8: IFT and cilia length regulation: plus end only turnover versus treadmilling
(*A*) Plus end only model of turnover. Individual microtubular doublets alternate between growth and shortening cycles. All alteration occurs strictly on the plus end. (*B*) Treadmilling model of turnover. New subunits are assembled at the microtubular plus end and simultaneously are old subunits are disassembled at the minus end. (adapted from Marshall & Rosenbaum, 2001)

5. Models of IFT regulation

For axoneme assembly IFT transport of many components, foremost tubulin is necessary (Craft et al., 2015). How cells control their cilia growth and length is still unclear. It appears that in many organisms ciliary growth is tightly linked to the cell cycle. Moreover, dynamics of the cilium differs across cilia types and species. In some cells, such as mammalian primary cilia or the cilia in Chlamydomonas, the organelles are rather dynamic, while in other, such as *Trypanosoma brucei*, is rather stable (Bertiaux et al. 2018). Hence, several models have been proposed to explain this complex process (reviewed by Lechtreck et al., 2017). In other organisms, ciliary growth is tightly linked to the cell cycle. Across species, the dynamic of the cilium differs.

The first of the models is the transport-limited model (Figure 9A). According to this model, the cells employs more IFT trains, when cilia grows, but then their amounts stars to decrease. The quantities of cargo load per one train is constant (Lechtreck et al., 2017). Nonetheless, recently amounts of IFT fluorescence intensity in the *Trypanosoma* were quantified, which showed a linear increase in number of IFT during axoneme elongation. The amount of IFT trains was constant per unit length of the axoneme (Bertiaux et al., 2018).

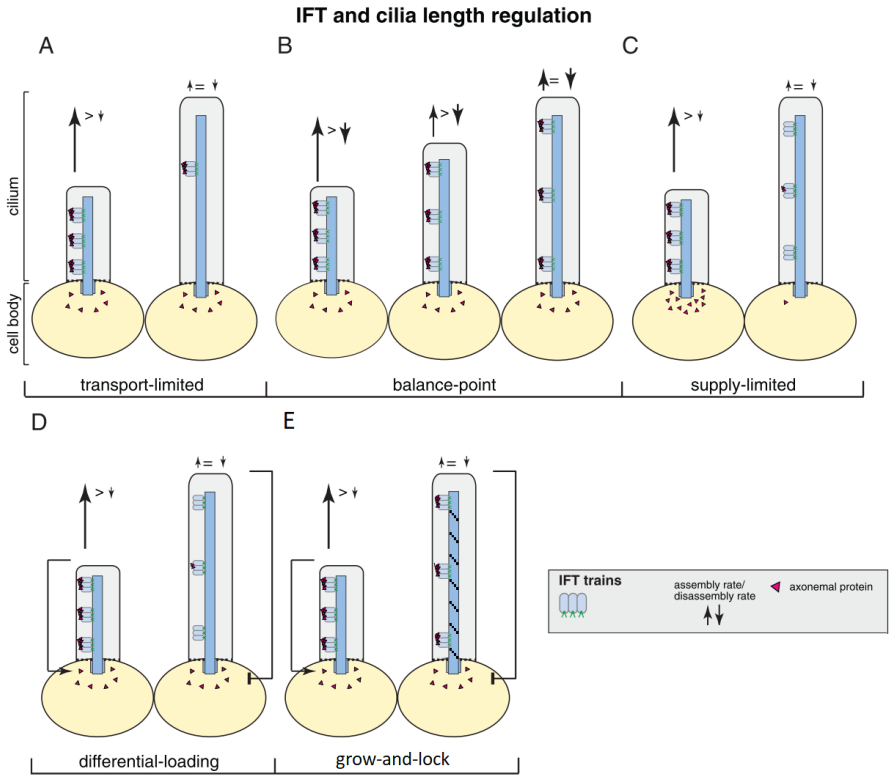

Figure 9: IFT and cilia length regulation
Five basic models for ciliary length control. The transport-limited model (*A*) shows more trains employed at the beginning of the ciliary growth and then a decrease in their amount. Balance-point (*B*) model suggests that the regulation occurs due the extending of the molecular motors must cover as the axoneme grows. The supply-limited pool model (*C*) proposes the limited amount of the building blocks available for the cilia assembly. When the pool gets drained, cilium cannot grow anymore. The differential-loading model (*D*) suggests, that the regulation factor is the ability of the cargo and IFT trains and to associate and disassociate The grow-and-lock model (E) postulates, that after a reaching the final length the locking event occurs, which modifies the cilium and prevents any further elongation (Bertiaux et al., 2018). (adapted from Lechtreck et al., 2017)

The second model is called the balance-point model (Figure 9B). In this model, the growth slows down because of increasing length of the axoneme. The train velocity is constant, but they need to overcome longer and longer distance. Disassembly of microtubules at the plus end is IFT dependent. This implies a regulation model, when the length regulation depends on a balance between tubulin assembly at the tip and its disassembly. The disassembly of axonemal tip is IFT independent. The growth therefor depends on the amount of transported cargo, which takes longer as the distant increase. Thus, the main role of IFT in full-grown is maintaining the balance between the disassembling the subunits of microtubules and assembling new subunits via transport of new building blocks at the tip (Marshall & Rosenbaum, 2001). On the other hand, differential interference contrast microscopy observation exposed, that the number of the IFT trains is not constant but differs with the ciliary length (Dentler, 2005). Nonetheless, the number of IFT trains does not have to be crucial. As a matter of fact,

the size of IFT trains often varies (Pedersen et al., 2006), which could explain previously reported irregularities. Changes in IFT train size could therefor control the assembly rate in the context of fluctuation of the axonemal length as postulated by the balance-point model (Engel, Ludington, & Marshall, 2009).

The third model, the supply-limited or the limiting pool model (Figure 9C) proposes that growth of the cilium is determine by axonemal precursor pool in the cell body. When this pool is exhausted, the growth stops. The regulation would take place on the protein production level. (Lechtreck et al., 2017). According to this model, if the material supply was limited, the pool would be draining, which would lead to lowering the concentration of this material in the cytoplasm. This mechanism would provide the decreasing velocity of the rate of incorporating of the subunits. This model can also explain, how the multi-ciliated cells can regulate the length of all its cilia. If the source of building blocks was limited, there would be restricted number of this structures allowed. New structure could not be assembled, until another cilium disassembles. The same rules would be possible to apply for similar structures such as centrioles (Goehring & Hyman, 2012). However, after comparison of fractionalized cell of the *Trypanosoma brucei* mutant in anterograde transport and wild type, it was not found significant difference of precursor pool. Hence is not likely, that the amount of tubulin precursors are the limiting factor causing shorter phenotype (Bertiaux et al., 2018).

The fourth model is known as the differential-loading or cargo-loading model (Figure 9D). This model suggests, that the axonemal length relies on the level of association of tubulin to IFT trains. The differential-loading model are supported by experiments with DRC4-GFP (subunit of dynein regulatory complex tagged with GFP) protein. Observation of this protein, as it is transported in the flagellum show dissociation from the IFT particles at various points along the cilium. Therefore, the cargo is not unloaded from IFT particles only at the tip but also along the whole cilium. After DRC4-GFP was unloaded from IFT particle, protein converted into stationary state. An explanation could be the diffusion of unloaded particles before docking into the axoneme. When the cilium is not assembled completely, the transported amount of the tagged cargo protein is increased. This suggest, that when cilium still grows, the amount of free docking sites is higher and with an increasing time their number is linearly reducing. Length of the period of the diffusion depends on accessibility of free binding sites. This model propose capability of the cell to sense shortened cilium and response through increased amount of loaded cargo until the intended length is reached (Wren et al., 2013).

The fifth model – the grow-and-lock model (Figure 9E) was based on observation, that amounts of IFT trains were increased as the axoneme got elongated (Bertiaux et al., 2018). Depletion of IFT kinesin in *Trypanosoma brucei* results in assembly of a shorter axoneme, which continues the elongation after cell division, but is incapable of reaching the regular length. This cannot be explained by a limiting pool.

The grow-and/lock model proposes “locking” (increased stability) of the cilium after cell division. This is supported by the observation in *Trypanosoma brucei* cells with temporally IFT depletion. As a test of this model the authors restored expression of kinesin-2 in cells with shorter flagella leading to a regular function of IFT; however, the flagellar length was unchanged. This shows that when the maturation of the cilium was complete, no further elongation can occur, despite abundance of the axonemal precursors. The grow-and-lock model proposes that axonemal length is regulated at the cell-cycle-dependent mechanism. The arrest of axonemal growth is activated by a specific signal preceding cytokinesis (Bertiaux et al., 2018).

The regulation of the ciliary length may also be provided by factors, which are not localized in the axoneme. The TZ could through its alternation serve as the control centre The TZ could regulate the influx of ciliary precursors which would partly correspond with the supply-limited model or transport limited model. The transitional fibres may rise the local concentration of both IFT and cargoes and due to this endorse the creation of IFT train complexes. The signal produced from non-fully-grown cilia could transform the behaviour of the basal body and transition zone causing higher quantity of loaded axonemal precursors to the IFT trains (Craft et al., 2015). Another possibility is regulation by a combination of these models.

6. Conclusion

Discovery of the intraflagellar transport finally answered the question how cilia are assembled, maintained and disassembled during the cell cycle. Although these organelles, mainly due an increased recognition of ciliopathies, drew recently a significant attention, there are still many outstanding unanswered questions, e. g. what the mechanism of organelle-length assessment and regulation is, factors influencing proper function of this machinery or details of the events at the tip, when the growth and turnover occur. Moreover, how is cargo bound to the IFT particles is still largely unknown.

Crucially, there is a significant level of variability in morphology and composition between various types of cilia. Hence, there may not be a single universal model explaining behaviour of all flagella, and different models or their combinations must be explored.

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